UNIVERSITÉ DE LAUSANNE – FACULTÉ DE MÉDECINE
CLINIQUE MÉDICALE UNIVERSITAIRE
Directeur: Professeur L.R. Freedman

TECHNICAL STUDY OF THE LEUCOCYTE MIGRATION INHIBITION TEST IN AGAROSE. APPLICATION TO PPD AND TO HEPATITIS B ANTIGEN.

THÈSE

présentée à la Faculté de Médecine de l'Université de Lausanne pour l'obtention du grade de docteur en médecine

par

PHILIPPE ERARD
Médecin diplômé de la Confédération Suisse
Originaire du Noirmont (BE)

TEX
Traitement du texte S.A.
Prilly
1975

UNIVERSITÉ DE LAUSANNE – FACULTÉ DE MÉDECINE
CLINIQUE MÉDICALE UNIVERSITAIRE
Directeur: Professeur L.R. Freedman

TECHNICAL STUDY OF THE LEUCOCYTE MIGRATION INHIBITION TEST IN AGAROSE. APPLICATION TO PPD AND TO HEPATITIS B ANTIGEN.

THÈSE

présentée à la Faculté de Médecine de l'Université de Lausanne pour l'obtention du grade de docteur en médecine

par

PHILIPPE ERARD
Médecin diplômé de la Confédération Suisse
Originaire du Noirmont (BE)

TEX
Traitement du texte S.A.
Prilly
1975

Université de Lausanne

Faculté de Médecine

Le Conseil de la faculté de médecine de l'Université de Lausanne, après avoir pris connaissance des rapports favorables de MM. les professeurs

P. MAGNENAT et D. GARDIOL

et sans se prononcer sur les opinions du candidat, autorise l'impression de la thèse intitulée :

" Technical study of the leucocyte migration inhibition test in agarose. Application to PPD and to hepatitis B antigen".

présentée par Monsieur Philippe ERARD

médecin diplômé de la Confédération Suisse

pour l'obtention du grade de Docteur en médecine.

Lausanne, le 26 février *19* 75

Le Doyen :

Prof. G. Candardjis

UNIV. LEIDEN BIBL.

REMERCIEMENTS

Ce travail a été exécuté dans la Division d'Immunologie et d'Allergie sous la direction du Dr P.C. Frei, PD. Pendant un an et demi nous avons bénéficié de son sens de la recherche clinique, de ses qualités d'organisateur et de sa disponibilité. Qu'il trouve ici l'expression de notre reconnaissance. Nous avons pu travailler dans un laboratoire où s'effectue une recherche rigoureuse dans un esprit de collaboration et nous tenons à remercier pour leur excellente aide technique, Mme G. Perruisseau, Mlle M. Ruckstuhl et M. R. Peitrequin.

Les Professeurs L.R. Freedman et P. Magnenat nous ont apporté un appui bienveillant et ont accepté de revoir le manuscrit de cette thèse. Nous les en remercions sincèrement.

Digitized by the Internet Archive
in 2026

https://archive.org/details/IA41546809_0071

in. exp. Immunol. (1974) **18**, 439–448.

TECHNICAL STUDY OF THE LEUCOCYTE MIGRATION INHIBITION TEST IN AGAROSE

APPLICATION TO PPD AND TO HEPATITIS B ANTIGEN

P. ERARD

Division d'immunologie et d'allergie, Département de médecine, Hôpital cantonal universitaire, Lausanne, Switzerland

(*Received* 1 *February* 1974)

SUMMARY

A modification of the leucocyte migration inhibition test, in which cells migrate in an agarose layer, was proposed by Clausen (1971). In the present study, this method was applied to PPD and hepatitis B antigen (HBAg), and its advantages were reassessed.

From a technical standpoint, the migration in a monolayer facilitated a study of the morphology of the migrating cells. The proportion of the different kinds of migrating leucocytes was calculated and expressed as a function of the distance of migration. On the whole, 92% of the migrating leucocytes were polymorphonuclears and 8% lymphomonocytes. Three to 10% of the incubated cells were shown to migrate in the agarose layer. The surface area of the controls (migration without inhibition) was sufficiently constant and large (on the average 13 mm^2) to allow a clear assessment of inhibition when antigen was added. In addition, the small requirement for antigen facilitated the use of this test in experimentation with purified HBAg.

Application of the technique to clinical problems revealed that, with PPD as antigen, a highly significant inhibition was obtained in PPD skin test-positive normal individuals ($0{\cdot}0025 < P < 0{\cdot}005$). But this inhibition was not significant ($0{\cdot}05 < P < 0{\cdot}1$) in PPD skin test-positive patients during the course of acute hepatitis B. With HBAg as antigen, a significant inhibition was obtained in individuals having recovered from acute hepatitis B. Such an inhibition was not demonstrable in individuals without a history of hepatitis, nor in patients during the course of the disease.

These results demonstrate the suitability of the leucocyte migration inhibition test in agarose for routine detection of cell-mediated immunity in man. They further suggest the development of cell-mediated immunity specific for HBAg after recovery from acute hepatitis B and impairment of this type of immune response to PPD during the acute phase of hepatitis.

Correspondence: Dr P. Erard, Division d'immunologie et d'allergie, Département de médecine, Hôpital antonal universitaire, CH 1011 Lausanne, Switzerland.

INTRODUCTION

Specific cell-mediated immunity can be detected *in vitro* by several methods (Bloom & Glade, 1971). In the leucocyte migration test, described by Søborg & Bendixen (1967), a total population of human leucocytes is incubated in a capillary tube in the presence of antigen and the size of their migration out of the capillary tube is measured. This test has been used in several clinical circumstances, but its significance is still a matter of controversy (Bloom *et al.*, 1973). In view of the lack of confidence in the reproducibility, Maini *et al.* (1973) performed standardization experiments on the capillary tube method, and proposed a standard technique for clinical application. Nevertheless, a modification of these methods which would be simpler, more reproducible and allow an easier study of the migrating cells is still strongly needed to study clinical conditions.

Clausen (1971) described a modification of the leucocyte migration test, in which human leucocytes were introduced into a well punched in an agarose gel layer and allowed to migrate between the gel and the bottom of the Petri dish. This method was used to demonstrate a constant inhibition of leucocyte migration in the presence of PPD in patients with positive PPD skin test (Clausen, 1971). Such an inhibition was also obtained with the supernatant from a culture of lymphocytes, which had been previously stimulated by PPD (Clausen, 1973) and by the supernatant of a mixed lymphocyte culture (Clausen, 1972).

In the present study, the phenomenon of leucocyte migration in agarose gel was examined further with particular attention directed to the nature of the migrating leucocytes. In addition, studies were conducted of the dynamics of this type of migration inhibition as a function of time.

Recovery from hepatitis B is a clinical model, in which the initiation of cell-mediated hypersensitivity can easily be observed, since this clinical condition is frequent and the responsible antigen well known (Dudley, Giustino & Sherlock, 1972; Frei, Erard & Zinkernagel, 1973). For this reason, the leucocyte migration inhibition test in agarose was applied to this model, using hepatitis B antigen (HBAg) as well as PPD as antigens. Results obtained in the presence of PPD were correlated with the delayed skin tests performed with this antigen. Results obtained with HBAg were correlated with the clinical evolution of hepatitis B.

MATERIALS AND METHODS

Patients

A total of forty-seven adults was investigated. Their skin reactivity to PPD was studied by intradermal tests. Individuals who did not show any skin reaction when injected with increasing amounts of tuberculin up to 0·1 μg, were considered PPD-negative. The skin reaction was considered as positive when the erythema was larger than 4 mm in diameter. These forty-seven individuals were divided into four groups.

Group a. Ten normal individuals (four males and six females) with positive skin test to tuberculin (average age 53 years). None of these individuals had a history of hepatitis. HBAg and the antibody against this antigen (HBAb) were not found in their serum by radioimmunoassay (RIA).

Group b. Nine normal individuals (three males and six females) with negative skin test to tuberculin (average age 36 years). None of them had a history of hepatitis. HBAg and HBAb were not found in their serum by RIA.

Group c. Fifteen individuals (eight males and seven females) with a history of HBAg ositive hepatitis between 2 and 37 months previously (average age 38 years). All were nically healthy and presented no sign of liver disease. HBAg was found to be negative by IA; transaminases, alkaline phosphatase, bilirubin and serum electrophoresis were normal. ll subjects of this group had a positive skin test to PPD.

Group d. Thirteen patients (five males and eight females) studied during the course of an ute hepatitis B, between 7 and 59 days after the beginning of icterus (average age 36 years). ll patients of this group had a positive skin test to PPD.

etection of HBAg and HBAb

All sera were tested by counterimmunoelectrophoresis (CIEP) using an antiserum of human igin (Immuno, Vienna), and, when positive, the titre of the antigen was measured. HBAg as also detected by RIA (Ausria-125, Abbott Laboratories, Radio-pharmaceutical oducts Division, Chicago) in all sera which were found to be negative on CIEP. The mmercial kit Ausria-125 was also used for the detection of HBAb, with the following odification: the antibodies adhering to the walls of the Ausria tubes were coated with 0·1 l of HBAg-positive serum. After 2 hr incubation at 45°C and five washings, 0·1 ml of the atients' sera to be tested was introduced into the tubes and incubated for 60 min at 45°C nd again washed five times. 0·1 ml of labelled HBAb was then added, and, after incubation nd washings, the bound radioactivity was measured. A decrease of a third of the radio- ctivity, as compared with the controls, was considered to indicate the presence of HBAb. his method did not allow the demonstration of antibodies in a serum in which the antigen as also present.

ntigens

HBAg was purified from the serum of a hepatitis patient, using a four-step procedure escribed elsewhere (Zinkernagel, Erard & Frei, to be published). Briefly, these four steps ere as follows: (1) ultracentrifugation or precipitation by ammonium sulphate; (2) filtra- on on Sephadex G-200; (3) electrophoresis on agarose-block; (4) ultracentrifugation on ucrose gradient. The antigen was then concentrated by vacuum dialysis in TC medium 199 Difco Laboratories, Detroit). The titre of the final preparation was found to be between :6 and 1:32, according to the batch when titred on CIEP, as previously mentioned. This orresponded to titres of 1:640 to 1:2560 when using RIA Ausria. No precipitation line in gar double diffusion was found, when using antiserum to whole human serum. On elec- onic microscopy, only 20 nm particles were seen.

Purified tuberculin (PPD), without preservative, was obtained from Statens Seruminstitut, openhagen.

eucocyte migration inhibition test

All experiments were performed under sterile conditions.

Preparation of leucocytes. Thirty millilitres of venous blood was mixed with preservative- ee heparin (Schuchardt, Munich) at a concentration of 12 u of heparin per millilitre of lood. Leucocytes were then separated by adding 7·5 ml of dextran 250, 6% (Pharmacia, Jppsala, Sweden). After 1 hr of sedimentation at 37°C, the supernatant was taken, centri- uged at 300 *g* for 10 min and washed three times in TC medium 199. The leucocytes were

then counted and the proportion between lymphocytes, neutrophils and eosinophils established. Red-cell contamination was expressed as percentage of the number of leucocytes. 20×10^6 leucocytes were then resuspended in 50 μl of each of the following solutions: (a) TC 199 and 10% foetal calf serum (FCS) (Microbiological Associates, Bethesda, Maryland); (b) TC 199 and 10% FCS and 25 μg of PPD; (c) TC 199, 10% FCS and purified HBAg at a final concentration of 1:3 on CIEP.

Preparaton of gel. Three hundred milligrams of agarose (Industrie Biologique Française, 92 Genevilliers, France) were heated at 100°C in 32 ml of distilled water and then incubated in a water-bath at 50°C. After having been brought to a temperature of 50°C, the following reagents were added to the agarose: 4 ml of FCS; 4 ml of 10× concentrated TC 199; 0·5 ml of TC bicarbonate 10% (Difco). The final product was a gel of 0·75% agarose and 10% FCS in TC 199, with a pH value of 7·4 when equilibrated with 2% CO_2. Two millilitres of the gel were then poured in each of eighteen Petri dishes (ϕ 35 mm, Falcon Plastics, Oxnard, California 93030). After the agarose had solidified, four wells (ϕ 2·5 mm) were punched.

Migration. Five microlitres of the leucocyte suspension (2×10^6 leucocytes) were introduced into each well using an automatic syringe (Eppendorf). Two dishes (eight wells) were filled with each suspension. Six dishes were thus necessary to test one patient. The preparations were then incubated for 18 hr at 37°C in a humid atmosphere containing 2% CO_2, so that the pH was constant.

Readings. After the incubation, the areas of migration were drawn on paper using a projection microscope (enlargement ×38) and measured by planimetry. Only the surface of dense migration was drawn. In ten experiments, the areas of migration were measured after 6, 18 and 24 hr of incubation. Since the exact limit of the surface of dense migration might be influenced by subjective appreciation, migration measurements were blind.

Calculation of the migration index. The mean of the eight identical migrations—minus the area of the well—was calculated. The migration index was calculated as: MI = [(mean of eight areas of migration with antigen)/(mean of eight areas of migration without antigen)] × 100. Values lower than 90% were arbitrarily considered as demonstrating an inhibition.

Study of the migrating cells. In ten experiments, which were performed in order to study in particular the nature of the leucocytes able to migrate in agarose, microscope slides were used instead of Petri dishes. After the incubation and after the areas of migration were measured as usual, the preparations were heat fixed: they were held over the flame of a Bunsen burner until the agarose melted and flowed freely from the slide. The slides were then stained with the May–Grünwald–Giemsa reagent. A microscopic examination (enlargement ×800) allowed the exact counting of each kind of leucocyte.

The proportion between these cells was determined in different concentric zones, starting at the centre and progressing to the periphery. The total number of migrating cells was then calculated.

Statistics. Groups of patients were compared using the Student's *t*-test for unpaired values.

RESULTS

Migration of leucocytes in agarose (without antigen)

In the ten experiments which were performed for the analysis of the leucocytes, the

verage total number of migrating cells was 133×10^3 (s.d. = 79×10^3). This value repre-ented about 3–10% of the number of the leucocytes introduced into the well.

Table 1 gives the proportion of the different kinds of leucocytes (a) in the suspension ntroduced into the well, (b) in the zone of dense migration and (c) in the zone of non-dense

TABLE 1. Morphological repartition of leucocytes, expressed as a percentage (±s.d.) in (a) a suspension of the cells introduced into the well before migration, (b) a zone of dense migration and (c) a zone of non-dense migration

Cells	Suspension before migration	Zone of:	
		Dense migration	Non-dense migration
Neutrophils	69±7·9	91±3·1	100
Eosinophils	5±2·6	1±0·6	0
Lympho-monocytes	26±5·3	8±2·4	0

The results are the mean of ten experiments.

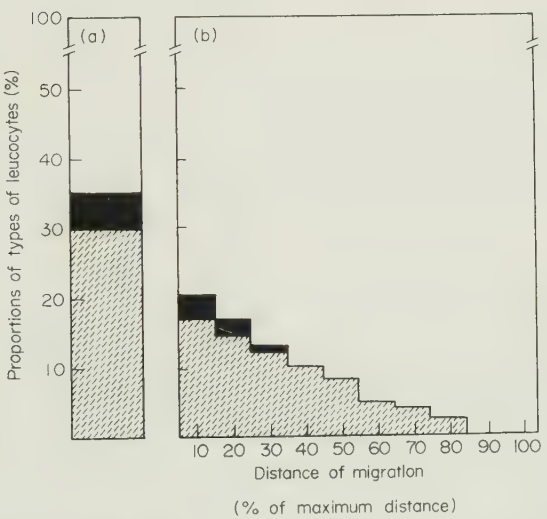

FIG. 1. Proportion of the neutrophils (□), eosinophils (■) and lympho-monocytes (▨), expressed as a percentage in (a) the suspension of cells before migration and (b) among the migrating cells (zone of dense migration). The proportion of the cells in the latter group is represented as a function of the percentage of the maximum distance of migration in each experiment. The results are the mean of ten experiments.

migration. Fig. 1 shows that the proportion of the cells within the zone of dense migration varied greatly according to the distance from the well. This distance was expressed as the percentage of the longest distance of migration. Eosinophils could not be seen further than 30% of the maximum distance. Lympho-monocytes could be seen as far as 80% of the

maximum distance, but their proportion was very low when counted so far from the well. On the whole, it was clear that the migrating cells consisted to a great extent of neutrophils. Among these latter cells, only segmented forms could be seen and the hypersegmented migrated the farthest.

In the forty-seven individuals, the average area of the control migration (without antigen) —minus the surface of the well—was 169·2 cm^2 (s.d. = 88·9) when enlarged by projection. This corresponds to a surface of 13 mm^2 (s.d. = 6·6 mm^2) on the preparation. In these experiments, the leucocyte suspension before incubation consisted on an average of 75·6% polymorphonuclears (s.d. = 8·6%) and 24·4% lymphocytes. A correlation could be demonstrated between the proportion of polymorphonuclears and the size of the area of migration ($r = 0{\cdot}43$, $0{\cdot}001 < P < 0{\cdot}01$). The contamination by erythrocytes was on the average 183% (s.d. = 114%) of the total number of leucocytes; it was shown that this contamination did not either influence the size of the area of migration ($r = 0{\cdot}05$).

TABLE 2. Leucocyte migration inhibition test in the presence of PPD

Normal individuals (group a, PPD-negative)			Normal individuals (group b, PPD-positive)			After recovery from acute hepatitis (group c, PPD-positive)			Acute hepatitis (group d, PPD-positive)		
93*	103†	112‡	148*	105†	71§	202*	178†	88¶	134*	131†	98**
175	189	108	101	49	49	295	198	68	131	109	83
112	124	112	187	114	60	110	98	89	151	102	61
123	124	101	175	79	45	191	123	64	168	172	102
138	128	92	124	101	81	187	66	35	124	123	100
89	83	95	65	51	78	104	82	83	224	222	99
313	338	108	162	120	74	99	107	108	115	159	138
257	246	96	124	85	68	104	111	116	83	70	84
91	96	106	121	92	76	80	41	50	132	128	96
			134	106	78	461	190	41	310	243	78
						427	386	90	183	204	111
						116	77	66	332	254	77
						256	170	66			
						165	96	58			

* Area of migration in TC 199 alone, enlarged 1300×, expressed in cm^2.
† Area of migration in TC 199+25 μg of PPD, enlarged 1300×, expressed in cm^2.
‡ MI. $\chi = 103{\cdot}3$; s.d. = 7·6.
§ MI. $\chi = 68{\cdot}0$; s.d. = 12·6; $0{\cdot}0025 < P < 0{\cdot}005$.
¶ MI. $\chi = 73{\cdot}0$; s.d. = 23·8; $0{\cdot}02 < P < 0{\cdot}025$.
** MI. $\chi = 93{\cdot}9$; s.d. = 19·6; $0{\cdot}05 < P < 0{\cdot}1$.

Migration in the presence of PPD

Table 2 allows the comparison of leucocyte migration inhibition in individuals with negative and with positive PPD skin tests. Individuals with negative skin tests were all normal (group a), whereas those with positive skin tests were either normal individuals (group b), individuals having recovered from acute hepatitis B (group c), or patients studied during the course of hepatitis (group d).

In group a, the MI was on the average 103·3% (s.d. = 7·6), and in all cases it was greater than 90. In contrast, all PPD-positive healthy individuals (group b) had a migration index

maller than 90; the difference between both groups a and b was highly significant ($0{\cdot}0025 < P < 0{\cdot}005$). In group c (recovery from hepatitis B), twelve out of fourteen patients had a migration index smaller than 90%, and, in three of them, the value was close to the limit; although the average migration index was higher than in group b, it was still significantly different from control group a ($0{\cdot}02 < P < 0{\cdot}025$). In group d (acute hepatitis), the migration index was smaller than 90 in only five out of twelve cases; compared to group a, the average migration index is not significantly different ($0{\cdot}05 < P < 0{\cdot}1$). In contrast, group d significantly differs from group c ($0{\cdot}01 < P < 0{\cdot}02$).

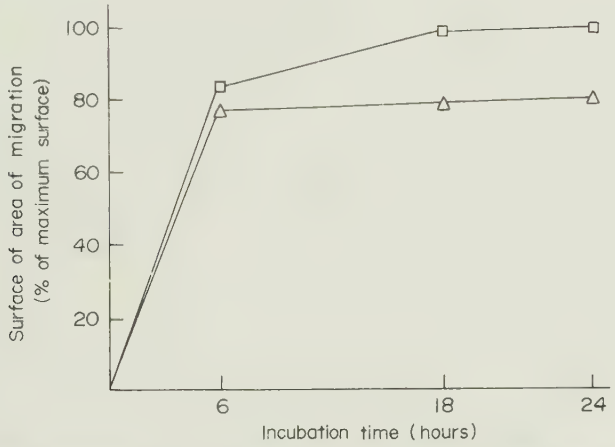

FIG. 2. Dynamics of migration. The surface of the area of migration is expressed as a percentage of the maximum surface without antigen and measured after 24 hr. This value is represented in function of the duration of incubation. The results are the mean of ten experiments. (□) TC medium 199 alone. (△) TC medium 199+25 μg of PPD.

Fig. 2 shows the evolution of the average migration and the average migration inhibition as a function of the duration of incubation in nine individuals with positive skin tests to PPD. In the control migration without antigen, the migration after 6 hr incubation represented 84% of the maximum migration after 24 hr. After 18 hr incubation, the area of migration corresponded to 99% of the maximum migration. In the presence of PPD, the general shape of the curve was very similar; the comparison with the control curve showed that the inhibition was clearly demonstrable after 18 hr only. Both curves show that the migration was negligible between the 18th and 24th hour.

Migration in the presence of HBAg

Table 3 shows that the migration index was on an average 105·5% (s.d. = 11·3) in control individuals, and that it was higher than 90% in all of them. In thirteen out of fifteen individuals who had recovered from hepatitis B, the index was smaller than 90% (average = 71·4, s.d. = 22·1). The difference between both groups was significant ($0{\cdot}0025 < P < 0{\cdot}005$). Table 3 also shows that no correlation could be demonstrated between the migration index and the time elapsed between the clinical recovery ($r = 0{\cdot}26$).

In only four of the thirteen patients of group d (acute hepatitis) was the index smaller than 90%. This group did not differ significantly from the controls ($0{\cdot}3 < P < 0{\cdot}4$). In contrast,

group d significantly differs from group c ($0{\cdot}0025 < P < 0{\cdot}005$). Table 3 also shows that no correlation could be demonstrated between the migration index and the duration of the disease ($r = 0{\cdot}24$).

TABLE 3. Leucocyte migration inhibition test in the presence of purified HBAg

Controls (groups a and b)			After recovery from acute hepatitis (group c)					Acute hepatitis (group d)				
148*	170†	115‡	202*	175†	87§	3**	+††	134*	175†	87¶	20‡‡	1/8§§
101	100	99	295	194	65	8	+	131	188	142	34	1/4
138	148	107	110	80	73	12	+	151	168	111	35	1/1
123	155	123	191	100	53	31	−	168	181	108	59	1/1
187	166	90	187	69	37	12	+	124	154	124	15	1/128
175	197	112	104	104	100	6	−	224	286	127	30	1/64
124	156	125	99	117	118	10	+	174	141	80	15	1/128
65	68	104	92	76	82	6	+	84	88	103	22	1/128
89	83	95	81	35	43	31	+	120	108	90	7	1/128
162	134	94	202	144	71	2	−	279	228	81	45	1/2
124	110	94	461	216	46	7		139	143	102	55	1/1
121	135	112	427	372	87	5	+	227	161	70	11	1/16
136	139	102	116	79	68	4	+	241	294	122	25	1/8
			256	208	81	37	−					
			165	100	60	2						

* Area of migration in TC 199 alone.
† Area of migration in TC 199 + purified HBAg.
‡ MI. $\chi = 105{\cdot}5$; s.d. $= 11{\cdot}3$.
§ MI. $\chi = 71{\cdot}4$; s.d. $= 22{\cdot}1$; $0{\cdot}025 < P < 0{\cdot}005$.
¶ MI. $\chi = 103{\cdot}6$; s.d. $= 7{\cdot}5$; $0{\cdot}3 < P < 0{\cdot}4$.
** Time in months elapsed since recovery.
†† Presence of HBAb by RIA.
‡‡ Time in days elapsed since the beginning of icterus.
§§ Titre of HBAg by CIEP.

HBAg and HBAb in serum

HBAg and HBAb could not be found in any of the sera from the normal control individuals (groups a and b).

HBAg was negative in all individuals of group c, whereas HBAb could be detected by RIA in nine of them (Table 3). By CIEP, the antibody could not be found in any of these cases.

HBAg could be found in the serum of all cases of group d (acute hepatitis) with titres between 1:1 and 1:128 (Table 3). In none of these cases could the antibody be detected by CIEP or RIA.

DISCUSSION

The leucocyte migration inhibition test according to Clausen (1971) was preferred to the usual capillary technique for the following reasons. (a) The test being more rapid and simple, the possibilities of errors were decreased. (b) The amount of antigen needed was very low; eight identical migrations can be performed with only 50 μl. This is of importance when

nly small quantities of purified antigen are available. (c) The areas of migration are large nough to permit reproducible results which are not influenced by the proportion of ymphocytes and polymorphonuclears, nor by red cell contamination. (d) Fixation, staining nd observation in monolayer of migrating leucocytes was easily performed.

The morphological study showed that the layer of migrating cells consisted of a great najority of segmented neutrophils. Since lympho-monocytes hardly participate in the nigration, it is probable that the cells sensitive to the inhibition phenomenon were poly-norphonuclears. This observation is at variance with the one of Rosenberg & David (1970), vho noticed in the capillary method an increase in lympho-monocytes among the migrating ells, as compared to the cell suspension before migration; the analysis of the zones of dense nd non-dense migrations suggested to these authors that only lympho-monocytes were nhibited. Since the leucocytes able to migrate in agarose appear not to be the same as those nigrating out of a capillary tube, the inhibition seems to concern different cells and the iological significance of both methods might not be identical. It is also possible that the ifficulty in studying migrating cells in a three-dimensional system (capillary tube) might xplain why their results were different from ours, which were obtained in a more simple wo-dimensional system. More recently, Egeberg, Bendixen & Nerup (1972) also found a nigher proportion of polymorphonuclears at the periphery of the migration.

A good correlation could be obtained between the PPD skin test and the inhibition of eucocyte migration by this antigen in the control group and, to a lesser degree, in the group of individuals after recovery from acute hepatitis. These observations suggest that this *n vitro* test represents delayed type hypersensitivity. Such a correlation could not be obtained during the course of acute hepatitis. An explanation for this fact could be a lepression of lymphocyte activity by hepatitis virus, as it was observed by other viruses (Olson *et al.*, 1968; Hall & Cantor, 1972; Haider *et al.*, 1973). In this hypothesis, lymphocyte lepression would perhaps be sufficient to prevent migration inhibition of polymorphonu-clears, but insufficient to prevent a positivity of the PPD skin test. One might also hypothe-size that the cytolysis of liver cells might liberate inhibitors of lymphokines.

The dynamics of migration show that inhibition was not demonstrable until 6 hr of incu-bation. After 6 hr, the area of migration in the presence of antigen remained the same, whereas migration continued in the control dishes without antigen. This delay in the demonstration of inhibition suggests that the phenomenon reflects cell-mediated immunity rather than the effect of preformed cytophilic antibodies, where inhibition of migration would have been observed early in the incubation period.

When HBAg was used as an antigen, a highly significant inhibition was observed in the group of patients who had recovered from acute hepatitis B. The inhibition was independent of the presence of circulating antibodies, as demonstrated by RIA (Table 3). Among the cases whose leucocytes were inhibited by HBAg, some had no detectable antibodies. Among the two individuals of this group, whose leucocytes were not inhibited by HBAg, one had detectable antibodies but not the other. These findings also suggest that the leucocyte migration inhibition test detects cell-mediated immunity rather than the effect of cell-bound antibodies. Although not quite identical, our results are in accordance with those of other authors, who used the capillary tube method (Dudley *et al.*, 1972; Reed *et al.*, 1974; Yeung Laiwah, Chaudhuri & Anderson, 1973). In a previous paper (Frei *et al.*, 1973), we showed that inhibition of leucocyte migration by HBAg could be demonstrated not sooner than 2 months and as late as 3 years after recovery from acute hepatitis B.

In the majority of the cases, inhibition by HBAg could not be demonstrated in the acute phase of acute hepatitis. Four hypotheses could be discussed to explain this observation. (a) Lymphocytes circulate in the patient's blood in the presence of an excess of HBAg, which saturates their binding capacity for antigen; they might thus be unable to react a few hours later *in vitro* with a relatively weaker concentration of antigen. (b) Lymphocytes able to react specifically with HBAg would be preferentially localized in the liver during the disease and the population collected at the periphery might be deprived of such lymphocytes. (c) For PPD, one may consider the role of immune depression by the virus or (d) the liberation of inhibitors of lymphokines.

ACKNOWLEDGMENTS

I wish to thank Dr Philippe C. Frei, who initiated and directed this study and Dr Rolf Zinkernagel, who purified the HBAg. This work benefitted by the technical assistance of Mrs Genevieve Perruisseau, Miss Marianne Ruckstuhl and Mr Roland Peitrequin and was financially supported in part by the Centre de Recherche sur les lymphomes malins (Professor Cardis, Lausanne).

REFERENCES

Bloom, B.R. & Glade, P.R. (1971) *In vitro Methods in Cell-mediated Immunity*. Academic Press, New York.

Bloom, B.R., Cepellini, R., Cerottini, J.C., David, J.R., Kunkel, H., Landy, M., Lawrence, H.S. Maini, R., Nussenzweig, V., Perlmann, P., Spitler, L., Rosen, F. & Zabriskie, J. (1973) *In vitro* methods in cell-mediated immunity: a progress report. *Cell. Immunol.* **6,** 331.

Clausen, J.E. (1971) Tuberculin-induced migration inhibition of human peripheral leucocytes in agarose medium. *Acta allergol.* **26,** 56.

Clausen, J.E. (1972) Migration inhibitory effect of cell-free supernatants from mixed human lymphocyte cultures. *J. Immunol.* **108,** 453.

Clausen, J.E. (1973) Migration inhibitory effect of cell-free supernatants from tuberculin-stimulated cultures of human mononuclear leukocytes demonstrated by two-step MIF agarose assay. *J. Immunol.* **110,** 546.

Dudley, F.J., Giustino, V. & Sherlock, S. (1972) Cell-mediated immunity in patients positive for hepatitis-associated antigen. *Brit. med. J.* **4,** 754.

Egeberg, J., Bendixen, G. & Nerup, J. (1972) Morphologic investigations on the leucocyte migration test (LMT). *Scand. J. Immunol.* **1,** 205.

Frei, P.C., Erard, P. & Zinkernagel, R. (1973) Cell-mediated immunity to hepatitis-associated antigen (HAA) demonstrated by leucocyte migration test during and after acute B hepatitis. *Biomedicine*, **19,** 379.

Haider, S., Continho, M. de L., Edmond, R.T.D. & Sutton, R.N.P. (1973) Tuberculin anergy and infectious mononucleosis. *Lancet*, **ii,** 74.

Hall, C.B. & Kantor, F.S. (1972) Depression of established delayed hypersensitivity by mumps virus. *J. Immunol.* **108,** 81.

Maini, R.N., Roffe, L.M., Magrath, I.T. & Dumonde, D.C. (1973) Standardization of the leucocyte migration test. *Int. Arch. Allergy*, **45,** 308.

Olson, G.B., Dent, P.B., Rawls, W.E., South, M.A., Montgomery, J.R., Melnick, J.L. & Good, R.A. (1968) Abnormalities of *in vitro* lymphocyte responses during rubella virus infection. *J. exp. Med.* **128,** 47.

Reed, W.D., Mitchell, C.G., Eddleston, A.L.W.F., Lee, W.M., Williams, R. & Zuckermann, A.J. (1974) Exposure and immunity to the hepatitis-B virus in a liver unit. *Lancet*, **i,** 581.

Rosenberg, S.A. & David, J.R. (1970) Inhibition of leucocyte migration: an evaluation of this *in vitro* assay of delayed hypersensitivity in man to a soluble antigen. *J. Immunol.* **105,** 1447.

Søborg, M. & Bendixen, G. (1967) Human lymphocyte migration as a parameter of hypersensitivity. *Acta med. scand.* **181,** 247.

Yeung Laiwah, A.A.C., Chaudhuri, A.K.R. & Anderson, J.R. (1973) Lymphocyte transformation and leucocyte migration inhibition by Australia Antigen. *Clin. exp. Immunol.* **15,** 27.